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Original article

An electrophoretic characterization of iron-transporting proteins in *Mannheimia haemolytica* A1

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Abstract

Iron-regulated outer membrane proteins (IROMPs) in *Mannheimia haemolytica* A1, which function as a receptor for complexes containing iron ions, are induced by iron deficiency in the growth environment of the bacteria. Densitometric analysis of SDS-PAGE separation showed expression of IROMPs of 71, 77, and 100 kDa in the case of bacteria grown in a medium with 2,2-dipyridyl. The electrophoregrams obtained in 2-DE separations confirmed the presence of protein fractions with these molecular weights and isoelectric points ranging from 5.4 to 6.4. The results of the study also confirmed the ability of *M. haemolytica* A1 proteins involved in iron uptake to induce a protective immune response. In Western blot with serum from convalescent calves naturally infected with *M. haemolytica* A1, distinct reactions were obtained for IROMPs of 71, 77, and 100 kDa.

Key words: *Mannheimia haemolytica* A1, iron-repressible outer membrane proteins, polyacrylamide gel electrophoresis, two-dimensional electrophoresis, Western blot

Introduction

Mannheimia haemolytica A1 is commonly identified with cases of bovine respiratory disease complex in calves (Wernicki et al. 1999, Akermann and Brodgen, 2000). The failure to develop an effective vaccine to prevent BRDC has drawn the attention of many research centres to the immunogenic proteins of *M. haemolytica* responsible for its pathogenesis. Among the wide range of antigens of this type, outer membrane proteins play a significant role. These include IROMPs (iron-repressible outer membrane proteins), which are involved in iron transport. They appear in the outer membrane of bacterial cells under conditions of iron deficiency and compete for iron with glycoproteins present in the organism. The ability to acquire iron *in vivo* is an important mechanism increasing the virulence of *M. haemolytica* (Gentry et al. 1986). Most pathogens produce highly efficient iron-acquisition systems that compete with transferrin and lactoferrin. One of these systems involves low-molecular-weight siderophores secreted by the bacterial cell and IROMPs with a high affinity for iron ions. They are induced by iron deficiency and function

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as receptors for iron-siderophore complexes. Within the *Pasteurellaceae* and *Neisseriaceae* families a different system has been identified, in which outer membrane receptor proteins bind specifically with the host's glycoproteins (Gray-Owen and Schryvers 1996).
In the case of *M. haemolytica*, this function is performed by proteins with molecular weights of about 31, 71, 77 and 100 kDa (Ogunnariwo and Schryvers 1990, Tabatabai and Frank 1999). These proteins function as receptors for complexes containing iron ions and play an important role in transporting them into the bacterial cell. Under *in vitro* conditions, the bacterial reaction to limited access to iron, taking the form of an increase in expression of IROMPs, occurs just 15 min. after incubation in an iron-deficient medium, attaining its maximum values after 30 min. (Deneer and Potter 1989, Roehrig et al. 2007, Puchalski et al. 2010). IROMPs must be rapidly induced so that the bacterial iron-acquisition system will be more efficient at the critical moment of infection, ensuring constant replenishment of intracellular iron reserves and, in consequence, colonization of host tissues. IROMPs of *M. haemolytica* are highly homologous in their amino acid sequences to corresponding proteins in *Actinobacillus pleuropneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, and *Neisseria gonorrhoeae* (Gray-Owen and Schryvers 1996). The importance of these proteins as virulence factors has been documented in many experiments. For instance, they have been found to be highly correlated with the virulence of *N. gonorrhoeae* for humans. *N. gonorrhoeae* mutants in which synthesis of these proteins was not observed were found to have no virulence (Cornelissen et al. 1998).

The aim of this study was to provide an electrophoretic characterization of proteins involved in transport of Fe ions in *Mannheimia haemolytica* A1. The immunogenic properties of these proteins in reactions with antibodies present in the serum of convalescent calves were also evaluated.

Materials and Methods

Strains of *M. haemolytica* A1 from the author's laboratory collection, isolated from calves with symptoms of BRDC, were used for the study.

A bacterial culture was grown in BHI medium for 18 h at 37°C. Expression of IROMPs was induced by adding 2,2-dipyridyl (final concentration 150 μM) to the medium. Outer membrane proteins were obtained by extraction with a 1% Sarkosyl (N-Lauroylsarcosine) solution from bacterial cells that had previously been exposed to ultrasound using a modification of a procedure by Morton et al. (1996), as described in a previous publication (Wernicki et al. 2002).

IROMPs were analysed using polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) and two-dimensional electophoresis (2-DE). For SDS-PAGE, protein samples prepared in a standard manner were separated in 12% resolving gel (Tris-HCl buffer, pH 8.8). Electrophoresis was carried out in Tris-Glycine chamber buffer at a constant voltage of 100V. SDS-PAGE Standards (Bio-Rad) with a range of 6.5 to 200 kDa were used as a molecular weight standard. The gels were stained with coomassie blue R-250 (Sigma) and after destaining of the background were analysed densitometrically using the Gel-Doc 2000 gel documentation system (Bio-Rad) and Quantity One software (Bio-Rad).

In the two-dimensional electrophoresis (2DE), in accordance with the procedure recommended by the producer of the Mini-Protean II 2-D Cell (Bio-Rad), modified by Urban-Chmiel et al. (2009), proteins obtained from *M. haemolytica* were dissolved in a sample solution (7 M urea, 2 M thiourea, 5% β-mercaptoethanol, 1% ASB-14, 1.6% Bio-Lyte 5/7 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte, 0.005% bromophenol blue). IEF separation (first dimension) was carried out in 75x1 mm glass capillary tubes filled with gel (9.2 M urea, 4% acrylamide/Bis, 20% Tryton X-100, 1.6% Bio-Lyte 5/7 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte, 0.01% ammonium persulfate, 0.1% TEMED). Separation was carried out at 500V for 10 min. and then at 750V for 3.5 hours. Gels with proteins separated in the first dimension were transferred to an equilibration buffer (0.05625 M Tris HCl pH 6.8, 2.3% SDS, 5% β-mercaptoethanol, 10% glicerol, 0.005% bromophenol blue). Second dimension separation was performed in 12% polyacrylamide gel according to Laemmli (1970). Molecular Weight Standards (Bio-Rad) with a range from 6.5 to 200 kDa were used, and 2-D SDS-PAGE Standards (Bio-Rad) were used to define isoelectric points. Comparative analysis of the gels following 2-DE, stained with coomassie blue R-250, was performed using PDQuest 2-D software (Bio-Rad).

The immunogenic properties of the IROMPs were evaluated by Western blot. Protein fractions obtained by SDS-PAGE were transferred to a nitrocellulose membrane (Supported Nitrocellulose membrane, 0.2 μm, Bio-Rad) according to the standard method of Towbin et al. (1979). Native serum obtained from convalescent calves from which *M. haemolytica* A1 had been isolated were used for the first antibody. The serum was diluted to 1:100 in TTBS buffer with 1% non-fat milk. These samples were incubated at 4°C for 12 hours. For the second antibody, rabbit antiserum containing horseradish-

-peroxidase-conjugated anti-bovine-IgG antibodies was used (Jackson ImmunoResearch). To induce a colour reaction, 1.4 chloronaphthol (Bio-Rad) was used as a substrate. Densitometric analysis of the images obtained was carried out using the Gel Doc 2000 gel documentation system (Bio-Rad) and Quantity One software (Bio-Rad).

Results

The electrophoresis image of the *M. haemolytica* A1 strain grown in standard conditions and in iron-limited conditions is presented in Fig. 1. Densitometric analysis of the separation showed that IROMPs with molecular weights of 71, 77, and 100 kDa were present in the bacteria grown on the medium with 2,2-dipyridyl. Two of these proteins, with molecular weights of 71 kDa and 100 kDa, were also synthesized in small quantities by the bacteria grown on the medium without a chelator.

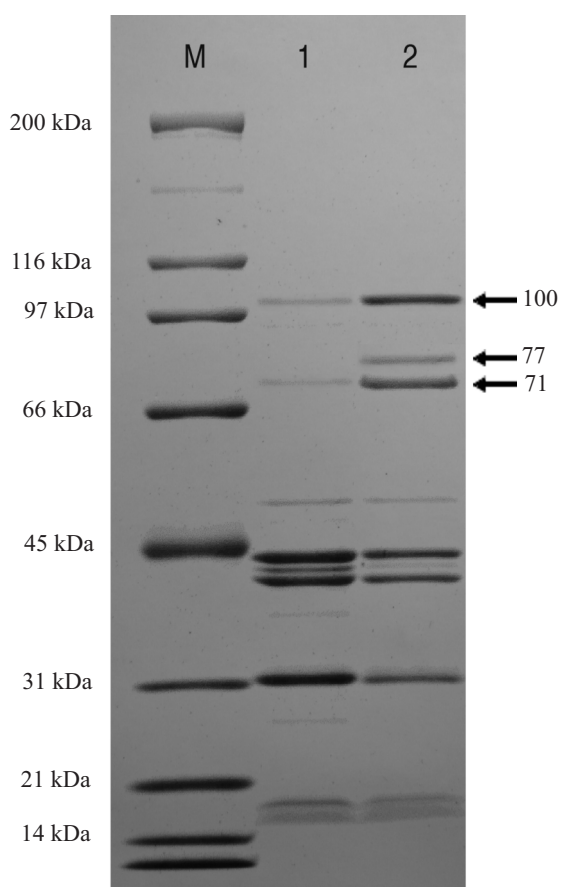

Fig. 1. Electrophoresis image (SDS-PAGE) of OMPs of *M. haemolytica* A1 grown in standard conditions and in iron-limited conditions. M – SDS-PAGE Standards (Bio-Rad). Lane 1 – *M. haemolytica* serotype 1 grown in standard conditions. Lane 2 – *M. haemolytica* serotype 1 grown in the presence of a chelator, with IROMPs of 71, 77, and 100 kDa indicated with arrows.

The electropherograms obtained in the 2-DE separations showed numerous protein fractions. The densest proteins were located in the 5.5-7.0 pH range and in the 30-100 kDa molecular weight range. Comparison of the electrophoregrams of the *M. haemolytica* A1 strain grown in iron-limited conditions (Fig. 3) and the strain grown in standard conditions (Fig. 2) showed additional proteins with a molecular weight of 100 kDa and an isoelectric point of 6.2, as well as proteins of 77 and 71 kDa with isoelectric points of 5.4 and 6.4, respectively.

Densitometric analysis of the image obtained on the nitrocellulose membrane revealed reactions between IROMPs and antibodies present in the serum of convalescent calves (Fig. 4). These reactions occurred in the case of proteins of 71, 77, and 100 kDa extracted from bacteria grown in iron-limited conditions. In the case of the bacteria grown in standard conditions, weak reactions were visible only for proteins of 71 and 100 kDa.

Discussion

A common element in bacterial iron-acquisition systems is the presence of specific IROMPs induced by iron deficiency. They act as receptors of complexes containing iron ions and are involved in the transport of these ions into the cell. Our study showed that *M. haemolytica* A1 also responds to an iron-poor environment with a change in the composition of outer membrane proteins. SDS-PAGE electrophoresis identified three proteins, with molecular weights of 71, 77, and 100 kDa. Of these, only the 77 kDa protein was not present in the bacteria grown in the presence of Fe ions. The other two proteins were present in cells grown with and without access to iron ions, but their concentration was significantly higher in the case of the medium with the chelator. Similar electrophoresis profiles were obtained by Deneer and Potter (1989). The present study also confirmed the presence of IROMPs of 71, 77, and 100 kDA. The results obtained did not enable 31 kDa proteins to be included among IROMPs, as Tabatabai and Frank had done (1999). While we did obtain such a band, its concentration was lower in the case of the bacteria grown in the presence of a chelator than in the medium with no iron deficiency.

Proteins of 100 and 71 kDa, designated as TbpA and TbpB, respectively, act together as a complex. They are subunits of the species-specific transferrin receptor. Genes for these proteins have been cloned, sequenced, and expressed in *E. coli* cells (Ogunnariwo et al. 1997). The role of TbpA and TbpB in inducing protection of calves against experimental

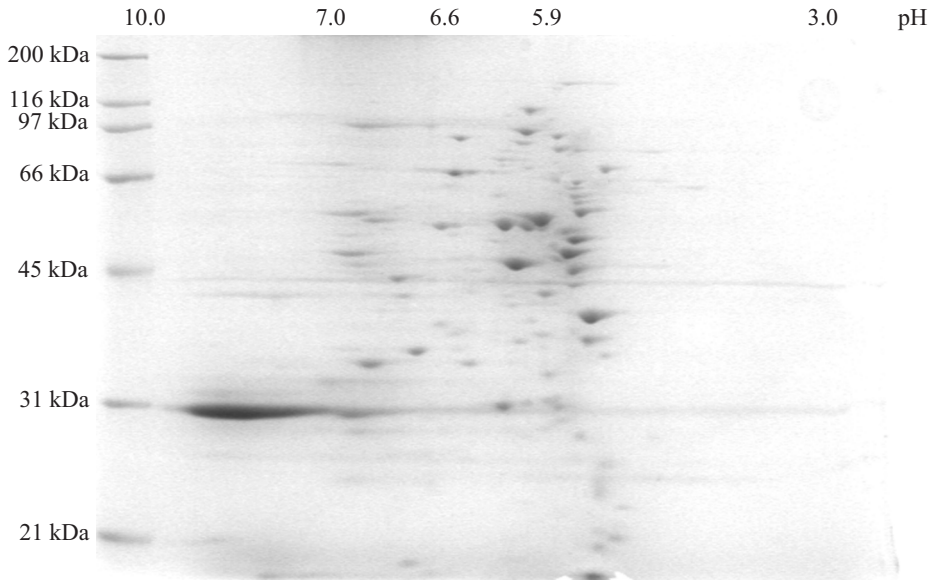

Fig. 2. Electrophoresis image (2-DE) of OMPs of *M. haemolytica* A1 grown in standard conditions.

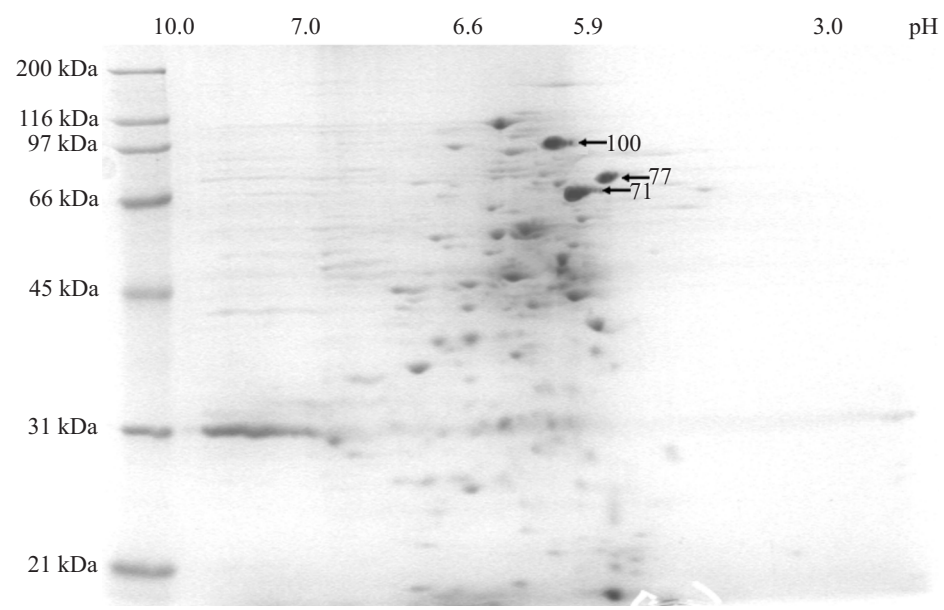

Fig. 3. Electrophoresis image (2-DE) of OMPs of *M. haemolytica* A1 grown in iron-limited conditions, with IROMPs of 71, 77, and 100 kDa indicated with arrows.

M. haemolytica infection has been documented by Potter et al. (1999). In their experiment, in which different combinations of the two proteins were used to construct vaccines used in 8-month-old feedlot calves, a high level of protective antibodies was noted for the recombinant TbpB, while a minimal humoral response was observed in the case of TbpA. According to the authors, the increase in protection resulting from the combined use of the two proteins can be explained by the possibility of induction of a cellular response by TbpA. This mechanism appears to be a synergistic complement to the protective effect of the humoral response, enabling more effective protection of the animals.

The results of the study confirmed that *M. haemolytica* A1 proteins involved in iron uptake are capable of inducing a protective immune response. In the immunoblot reaction with serum from convalescent calves naturally infected with *M. haemolytica*, distinct reactions were obtained for IROMPs of 71, 77, and 100 kDa. Similar results had been obtained by Deneer and Potter (1989) and Confer et al.

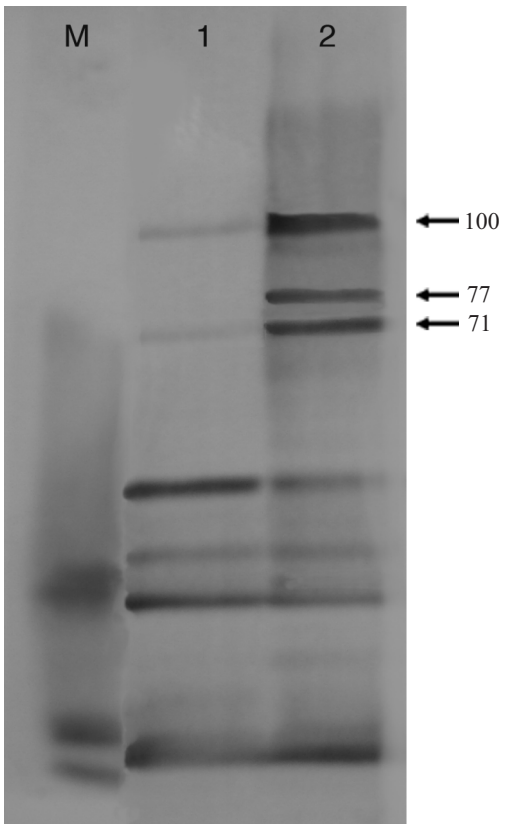

Fig. 4. Western blot of OMPs of *M. haemolytica* A1 grown in standard conditions and in iron-limited conditions with antibodies present in the serum of convalescent calves. M – Kaleidoscope Prestained Standards (Bio-Rad). Lane 1 – *M. haemolytica* serotype 1 grown in standard conditions. Lane 2 – *M. haemolytica* A1 grown in the presence of a chelator, with IROMPs of 71, 77, and 100 kDa indicated with arrows.

(1995), who demonstrated that vaccination of calves with live *M. haemolytica* A1 cells stimulates a humoral response to IROMPs of 71 and 77 kDa. Vaccination with outer membrane fractions enhanced with IROMPs isolated from *M. haemolytica* strains was found to stimulate a significant increase in antibodies against proteins of 71 kDa. The live vaccines stimulated only a slight increase in antibodies against 100 kDa proteins. The results of the present study and those obtained by Confer et al. (1995) may appear to be contradictory, but in both cases antibodies against proteins of 100 kDa were noted. According to Confer et al. (1995), any differences noted between the two studies in the intensity of binding of antibodies with 100 kDa IROMPs depend on the means of exposure to live strains of *M. haemolytica*. In the present study, the calves were infected naturally, while the results obtained by Confer et al. (1995) were the effect of vaccination. This may have caused rapid binding of TbpA with transferrin during an acute inflammatory reaction, decreased the availability of this protein to cells involved in the development of the immune response, and in consequence, led to insignificant induction of specific antibodies.

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